

Title - Overproduction of individual gas vesicle proteins perturbs flotation,
antibiotic production and cell division in the enterobacterium *Serratia* sp.
ATCC39006

Short title – Overexpression of gas vesicle proteins in *Serratia* sp. ATCC39006

Authors - Rita E Monson¹, Yosuke Tashiro² and George PC Salmond^{1*}

Address

1. Department of Biochemistry, University of Cambridge, CB2 1QW, Cambridge
United Kingdom

2. Applied Chemistry and Biochemical Engineering Course, Department of
Engineering, Graduate School of Integrated Science and Technology, Shizuoka
University, Hamamatsu 432-8561, Japan.

*For correspondence email gpcs2@cam.ac.uk

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Abstract

Gas vesicles are intracellular proteinaceous organelles that facilitate bacterial colonization of static water columns. In the enterobacterium, *Serratia* sp. ATCC39006, gas vesicle formation requires the proteins GvpA1, GvpF1, GvpG, GvpA2, GvpK, GvpA3, GvpF2, GvpF3 and the three gas vesicle regulatory proteins, GvrA, GvrB and GvrC. Deletion of *gvpC* alters gas vesicle robustness and deletion of *gvpN* or *gvpV* results in small bicone vesicles. In this work, we assessed the impacts on gas vesicle formation when each of these 14 essential proteins was overexpressed. Overproduction of GvpF1, GvpF2, GvrA, GvrB or GvrC all resulted in significantly reduced gas vesicle synthesis. Perturbations in gas vesicle formation were also observed when GvpV and GvpA3 were in excess. In addition to impacts on gas vesicle formation, overproduction of GvrA or GvrB led to elevated biosynthesis of the tripyrrole pigment, prodigiosin, a secondary metabolite of increasing medical interest due to its antimalarial and anticancer properties. Finally, when GvpG was overexpressed, gas vesicles were still produced, but the cells exhibited a growth defect. Further analysis showed that induction of GvpG arrested cell growth and caused a drop in viable count, suggesting a possible physiological role for this protein linking gas vesicle biogenesis and binary fission. These combined results demonstrate that the stoichiometry of individual gas vesicle proteins is crucially important for controlled organelle morphogenesis and flotation – and provides evidence for the first link between gas vesicle assembly and cell division, to our knowledge.

Introduction

Gas vesicles are intracellular proteinaceous structures that facilitate flotation throughout aquatic niches in a many different microorganisms (Pfeifer 2012). These hollow cylindrical structures are permeable only to dissolved gas in the medium and thus gas vesicles reduce the density of bacterial cells, allowing them to fully colonize a static water column through flotation (Walsby 1994). Gas vesicles are sensitive to pressure and production is often regulated in response to environmental and nutritional inputs from the organism's surroundings (Hechler & Pfeifer 2009; Pfeifer et al. 2002; Tashiro et al. 2016; Ramsay et al. 2011). To date, all gas vesicles identified are composed of the small hydrophobic protein GvpA, which self-assembles into a ribbed array structure forming the gas vesicle walls (Buchholz et al. 1993; Hayes et al. 1988; and reviewed extensively in Walsby 1994). The protein GvpC forms a mesh structure covering the surface of the vesicle, thereby providing additional strength (Dunton et al. 2006; Buchholz et al. 1993; Offner et al. 1996). The specific functions of the remaining proteins are largely unclear, though some have been shown to associate with gas vesicles or form minor components of the structure (Tavlaridou et al. 2014; Tavlaridou et al. 2013; Xu et al. 2014).

The genetic clusters responsible for gas vesicle production have been widely reported in Archaea, cyanobacteria and recently in the enterobacterium *Serratia* sp. ATCC39006 (S39006)(van Keulen et al. 2005; Mlouka et al. 2004; Ramsay et al. 2011; Halladay et al. 1993; Dunton & Walsby 2005). S39006 is a Gram-negative bacillus that produces the intracellular, red tripyrrole prodiginine antibiotic, 2-methyl-3-pentyl-6-methoxyprodiginine (prodigiosin; Williamson et al. 2006) and the β -lactam antibiotic, 1-carbapenem-2-em-3-carboxylic acid (a carbapenem; Coulthurst et al. 2005). Prodiginines have anticancer,

immunosuppressant and antimalarial properties and are of interest due to their pro-apoptotic activities (Hsieh et al. 2012; Marchal et al. 2014; Espona-Fiedler et al. 2012; Lu et al. 2012; Liu et al. 2013). S39006 is also a plant pathogen, that produces plant cell wall degrading enzymes such as pectinases and cellulases (Fineran et al. 2007; Fineran et al. 2005), but is also capable of killing the nematode worm, *Caenorhabditis elegans* (Coulthurst et al. 2004; Wilf et al. 2011). Many of these phenotypes, including production of gas vesicles, are regulated in a cell concentration-dependent manner through the SmalR quorum sensing system (Fineran et al. 2005; Ramsay et al. 2011). Finally, in addition to movement due to the presence of gas vesicles, S39006 can swim using flagella and can swarm across the surface of media facilitated by the production of a biosurfactant (Williamson et al. 2008).

In S39006, the gas vesicle genetic locus contains nineteen genes arranged in two operons (Figure 1), the first starting with *gvpA1* (an orthologue of *gvpA*) and the second with *gvrA* (a regulator of gas vesicle synthesis in S39006). The genetic cluster contains three closely related isoforms of GvpA, designated GvpA1, A2 and A3 and three isoforms of GvpF, designated F1-F3. Recently, we determined that 11 of the nineteen genes in the genetic locus were required for robust gas vesicle synthesis (Tashiro et al. 2016). That is, when any one of these genes was removed, either production of gas vesicles was abolished or, in the case of *gvpV* or *gvpN*, only small bicone gas vesicles were produced and these never developed into larger, cylindrical, mature forms. Additionally, in a *gvpC* mutant, while gas vesicles were produced, they were significantly less robust under pressure, suggesting that GvpC_{S39006} acts to strengthen gas vesicles, as it does in other systems (Tashiro et al. 2016). To demonstrate that, in each case, it was loss of the specific protein that prevented gas vesicle

production, we expressed the wild type genes from a plasmid in the cognate mutants to show restoration of gas vesicle production. This complementation confirmed genetically that the plasmid-based copy of the protein was expressed and functional in S39006.

While analysis of individual gene deletion mutants defined whether the corresponding proteins were essential for gas vesicle formation, it has been reported in other systems that stoichiometry of individual proteins is also important for gas vesicle production (Tavlaridou et al. 2013; Chu et al. 2011; Shukla & DasSarma 2004), though this has not been examined in S39006. For example, a recent study from *Halobacterium salinarum* demonstrated that overexpression of GvpG and GvpH resulted in a significant decrease in cells containing gas vesicles (Tavlaridou et al. 2013). Further, overexpression of GvpM reduced the number of cells containing gas vesicles; with producing cells showing reduced vesicle numbers but larger organelles (Tavlaridou et al. 2013).

As we had previously demonstrated that plasmid-based expression of each essential gas vesicle protein was capable of complementation (Tashiro et al. 2016), we decided to examine further the impacts of overexpression of each protein essential for gas vesicle production in wild type S39006.

Materials and Methods

Bacterial strains, plasmids and growth conditions

All bacterial strains, plasmids and bacteriophages used are listed in Table 1. *Serratia* sp. ATCC39006 LacA was used as a wild type background in all experiments. Overnight cultures of S39006 and *E. coli* strains were grown in lysogeny broth - Lennox (LB; 10 g tryptone l⁻¹; 5 g NaCl l⁻¹; 5 g yeast extract l⁻¹) in sealed plastic universals or LB agar (LBA) plates (1.5%) at

30°C and 37°C respectively. When necessary, strains were supplemented with ampicillin (100 µg ml⁻¹) or chloramphenicol (25 µg ml⁻¹). Where indicated, the inducer Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added, in different concentrations, to plates and liquid culture. Generalized transduction with bacteriophage φOT8 was used to move plasmids between S39006 strains (Evans et al. 2010).

Growth studies were performed in 250 ml flasks containing 25 ml LB, inoculated to an initial OD₆₀₀ of 0.05 and grown under aerobic conditions with shaking at 215 rpm. Under microaerophilic conditions, the same culture conditions were used but 25 ml of sterile mineral oil was placed on top of the LB, flasks were then shaken at 80 rpm to restrict O₂ availability. During growth studies, the OD₆₀₀ was ascertained by measurement on a Helios Zeta spectrophotometer and viable colony counts were determined by serial dilution and plating onto LBA.

Plasmid construction

The plasmid pQE80-GvpG_{domain} was constructed by amplifying the sequence encoding the GvpG conserved domain region using oligonucleotides oREM602 (5' - CCCGAATTCGCGCTGGTACTCTCTCTGACTGAG - 3') and oREM603 (5' - CCCGGATCCTCA TTCATCCAGTGCGTCTAATCTATC - 3') by PCR. This fragment was digested with *Bam*HI/*Eco*RI and ligated with compatibly digested pQE80-*oriT* to create pQE80-GvpG_{domain}. The recombinant plasmid sequence was confirmed by sequencing (GATC Biotech).

Gas vesicle formation assays

Assessment of gas vesicles on plates and flotation assays in liquid culture were performed largely as described previously (Ramsay et al. 2011; Tashiro et al. 2016). Briefly, for plate

based assays, a normalized number of cells were spotted and dried onto LBA, and incubated at 30°C. The patch coloration, shape and opacity was assessed after one, two or five days of growth. Flotation assays were performed in 5 ml overnight cultures, grown in 25 ml sealed plastic universals on a roller wheel at 30°C. Cultures were then left to settle on a level surface for 48 hours and visually assessed for colonization of the air liquid interface.

Microscopy

Phase contrast microscopy (PCM) and Transmission electron microscopy (TEM) images were taken as described previously (Tashiro et al. 2016; Ramsay et al. 2011). PCM images were processed using ImageJ (Schneider et al. 2012) and a scale bar added afterwards (calibrated at 22 pixels equal to one μm). TEM samples were stained with 2% uranyl acetate before imagine on a Tecnai G2 TEM, with an attached AMT XR60B camera in the Cambridge University Advanced Imaging Facility.

β -glucuronidase assay

Cultures were grown for 14 hours, 100 μl samples removed, frozen at -80°C. They were subsequently processed as described previously (Monson et al. 2015).

Prodigiosin assay

Prodigiosin production was quantified largely as described previously (Williamson et al. 2005). Briefly, after 14 hours of growth, one ml samples were taken, centrifuged, and the pellet frozen at -80°C. Prodigiosin was extracted from the samples and presented as $A_{534} \text{OD}_{600}^{-1.55}$.

Pressure nephelometry

Pressure nephelometry on S39006 was performed as described in Tashiro *et al.* 2016, and cultures were grown in LB carrying the indicated concentration of IPTG.

Bioinformatics

Domain architecture of GvrA, GvrB, GvrC and GvpG was determined by searching the pfam database (Finn et al. 2015). Alignments of amino acid sequences were performed using Clustal Omega (Sievers et al. 2011). Phylogenetic trees were generated using the following settings in NCBI: Fast Minimum Evolution, Max Sequence Difference of 0.85 and Grishin protein distribution. Trees were plotted using NJPlot (Perrière & Gouy 1996).

Results

Overexpression of some GV-associated proteins perturbs GV formation

Previously, we showed that deletion of any one of *gvp-A1, A2, A3, F1, F2, F3, C, N, V, G, gvr-A, B* or *C* resulted in impaired biosynthesis of gas vesicles - either in terms of size, strength or production. We were able to complement the deletion mutants by expression of the corresponding protein from a plasmid and so were confident that *in trans* plasmid-based expression of each of these proteins, produced a functional protein. Nonetheless, a recent study demonstrated that perturbing the relative amounts of individual proteins, can impact on gas vesicle production (Tavlaridou et al. 2013). We expressed each essential gas vesicle protein from a plasmid within wild type S39006. This plasmid system (pQE80-*oriT* based) induced expression on addition of IPTG. For each overexpression strain, we assessed gas vesicles on plates with no induction, mid-level induction (0.1 mM IPTG), and full induction (1 mM IPTG). Gas vesicles refract light, and thus colonial opacity was used as a facile monitor

of gas vesicle production in individual colonies (Walsby 1994; Ramsay et al. 2011). Furthermore, under PCM, intracellular groups of gas vesicles produce phase bright “gas vacuoles” which are easy to identify (Ramsay et al. 2011). Using these two techniques, we observed that overexpression of GvpF1, GvpA3, GvrA, GvpF2, GvrB and GvrC resulted in little or no gas vesicle production (Figure 2). An excess of GvpA1, GvpC, GvpN, GvpV, GvpA2, GvpK and GvpF3 did not visibly alter gas vesicle formation.

During these experiments, we noted that patches of strains overexpressing GvpG were slow growing, although GV's were produced in these strains (see Figure 2 and following sections). PCM analysis also showed that overexpression of GvpV resulted in a significant number of cells with no gas vesicles (Figure 2). After extended incubation (five days instead of two) of the colonies (Supplemental Figure 1) while we still observed that gas vesicle production was perturbed as above, the patch morphology of cells overexpressing GvpV was mottled and showed a bulls-eye pattern where gas vesicles (more opaque parts) were visible on the edges. Furthermore, translucent cells were visible towards the middle of the patch overexpressing GvpV. When cells from these older patches were observed by PCM, a significant population did not appear to produce gas vesicles (Supplemental Figure 1). A similar pattern of morphology was observed when GvrA was overexpressed at the highest levels, the outer ring of the patch appeared white, suggesting that cells in this area were expressing gas vesicles but without prodigiosin.

Colony or patch opacity and PCM analysis are indicators of gas vesicle biosynthesis. However, microbes are known to grow differently on agar plates than in liquid medium (Mikkelsen et al. 2007; Jeanson et al. 2015). As gas vesicles facilitate flotation in aquatic environments, we also wanted to examine gas vesicle production in liquid culture. To assess

whether perturbation of individual gas vesicle genes affected flotation, we overexpressed each gene required for gas vesicle synthesis in liquid culture and observed flotation of cells in liquid culture (Figure 3). Our results were similar to those seen on plates (Figures 2 and 3). Flotation was not observed when GvpF1, GvpF2, GvrA, GvrB and GvrC were overexpressed. However, gas vesicles were visible in a small population of cells overproducing GvpF2, suggesting that the lack of flotation was likely due to the majority of cells lacking any visible phase bright structures. Additionally, we observed a much more pronounced phenotype for cells overexpressing GvpA3 than we saw on plates. Cells were unable to float and almost no cells containing gas vesicles were visible by PCM (Figure 3). Furthermore, in liquid culture, while cells expressing GvpG appeared to float, the cells appeared more red (Figures 2 and 3), suggesting that an excess of GvpG impacts on the production of prodigiosin.

Overexpression of GvrA, GvrB or GvrC impacts *gvpA1* transcription

The gas vesicle gene cluster in S39006 is divided into two operons (Figure 1). During our earlier experiments (Tashiro et al. 2016), we found that overproduction of any one of the three regulatory proteins GvrA, GvrB or GvrC, and all regulatory proteins in the second operon, resulted in almost no visible gas vesicles on either plates or in liquid media (Figure 2 and 3, see 1mM IPTG conditions). GvrA, GvrB and GvrC all contain conserved motifs that suggest they form parts of a two-component system. GvrA contains a response regulator receiver domain (pfam00072), an AAA+ ATPase domain (pfam00158), characteristic of a σ^{54} interaction domain and helix-turn-helix motif (pfam02954) characteristic of bacterial regulatory proteins such as Fis. GvrB contains a PAS domain (pfam13426), a histidine sensor kinase domain (pfam00512), again characteristic of two component systems, and a histidine kinase-like ATPase domain (pfam02518). In contrast, GvrC contains only a single response

regulator receiver domain (pfam00072; summarized in Figure 4a). Additionally, when performing PSI-BLAST searches, the receiver domains between GvrA and GvrC were sufficiently similar to identify one when searching for the other. When aligning the sequences, we noted that the predicted dimerization interface (amino acid residues KPF) is conserved between both proteins (Supplemental Figure 2a). Furthermore, a phylogenetic analysis for each of GvrA, GvrB and GvrC revealed that, while they were conserved across many bacterial species, they were not found in other gas vesicle producing organisms such as the archaeon *Halobacterium salinarum* PHH1. Additionally, in *Burkholderia* sp., only GvrA has been identified so far but not GvrB or GvrC (Supplemental Figure 2), although, to date, there have not been any published reports of *Burkholderia* sp. synthesizing gas vesicles naturally.

We were surprised to find that an excess of GvrA/B/C resulted in almost no gas vesicle formation as, in a previous study, we demonstrated that deletion of any one of *gvrA*, *gvrB* or *gvrC* abrogated gas vesicle production. The latter effect was due to a decrease in *gvpA1* transcription, suggesting that all three were involved in activating *gvpA1* transcription (Tashiro et al. 2016). However, even leaky expression of *gvrC* from the plasmid alone (without induction) was enough to repress gas vesicle synthesis (Figures 2 and 3). Taken together, these results suggested that even small changes in the transcription levels of the regulatory genes *gvrA*, *gvrB* and *gvrC*, altered gas vesicle production. However, we were unsure if excess GvrA, -B or -C diminished *gvpA1* transcription or increased *gvpA1* transcription leading to a decrease in gas vesicle formation, due to a stoichiometric imbalance of particular gas vesicle proteins, such as GvpF1 (Figures 2 and 3). To determine which hypothesis was correct, we examined activity of a *gvpA1::uidA* reporter fusion when

GvrA, -B, -C production was induced. In this strain, transcription of the *gvpA1* promoter can be assessed by quantitatively by measuring activity of the UidA protein. Under both aerobic (Figure 4b) and microaerophilic (Figure 4c) conditions, expression of the *gvpA1::uidA* fusion decreased when any one of GvrA, GvrB or GvrC was overproduced. In the case of GvrC, even the presence of the uninduced plasmid construct caused a significant decrease in expression of the *gvpA1::uidA* fusion when compared to the empty vector control (Figures 4b and c). These results were consistent with our earlier observations of gas vesicles in colonies or in liquid flotation assays, and confirmed that an excess of any one of the three regulatory proteins decreased gas vesicle production by affecting transcription of the *gvpA1* operon.

Overexpression of GvrA and GvrB increases production of prodigiosin

While performing experiments examining the impact of an excess of GvrA and GvrB, we noticed that the cultures appeared to produce more of the intracellular pigment, prodigiosin. Prodigiosin production is tightly regulated in S39006 and, as with production of gas vesicles, is regulated in response to cell density²⁶. However, no previous studies have identified genes in the gas vesicle operons as regulators of prodigiosin. To determine whether an excess of gas vesicle regulatory proteins impacted prodigiosin synthesis, we examined production under inducing and non-inducing conditions. We found that an increase in GvrA led to a three-fold increase in prodigiosin production, while induction of GvrB led to a two-fold increase in prodigiosin production. Overexpression of GvrC had no discernible effect on prodigiosin production (Figure 4d). These results suggest that, in addition to their regulatory role in gas vesicle production, GvrA and GvrB, when in excess, also regulate the production of secondary metabolites such as prodigiosin.

Excess GvpC does not alter gas vesicle strength

Previously, many reports have demonstrated the role of GvpC in reinforcing gas vesicle strength (Dunton & Walsby 2005; Dunton et al. 2006). GvpC is thought to form a reinforcing mesh on the surface of the gas vesicle (Dunton et al. 2006; Buchholz et al. 1993). Furthermore, in *Anabaena*, it has been shown that when gas vesicles, stripped of GvpC, were subsequently saturated with GvpC, they were not stronger than when originally isolated (Buchholz et al. 1993). Thus, excess GvpC may not necessarily provide more strength to existing gas vesicles. S39006 mutants of *gvpC* produced gas vesicles but they collapsed under significantly less pressure than those produced in wild type cells, consistent with the view that GvpC was acting as a strengthening protein (Tashiro et al. 2016). Overexpression of GvpC did not appear to alter production of gas vesicles or flotation (Figures 2 and 3). However, we wondered if increasing GvpC might alter the gas vesicle critical collapse pressure. Using pressure nephelometry, we examined the robustness of gas vesicles under increasing pressure but found that there was no difference in critical collapse pressure upon overexpression of GvpC (Figure 5a).

Overexpression of GvpV results in cells with large amounts of small bicone vesicles

A S39006 mutation in either *gvpV* or *gvpN* results in cells containing only small bicone vesicles. These observations were interesting as *gvpV* has not been identified in the well-studied archaeon *H. salinarum* PHH1, while *gvpN* has been identified in a wide array of other organisms (Englert & Pfeifer 1993; Tashiro et al. 2016; Englert et al. 1992; Horne et al. 1991). We postulated earlier that GvpV and GvpN might act as molecular chaperones required for the development of mature cylindrical vesicles from the bicones seen in the early stages of the morphogenetic assembly pathway in S39006 (Tashiro et al. 2016). In our initial experiments, increased expression of GvpN had no obvious impacts on gas vesicle

formation. In contrast, we noticed a subtle decrease in the amount of gas vesicles present when we overexpressed GvpV. This phenotype became more pronounced on extended incubation and, after five days, patches overexpressing GvpV had a bulls-eye appearance, and PCM analysis revealed a mixture of cells with and without gas vesicles (Supplemental Figure 1). To assess cultural heterogeneity, we examined these cells by transmission electron microscopy (TEM) to see if the apparent lack of gas vesicles (as scored by PCM) was the result of the complete absence of vesicles or perhaps simply the presence of only small bicone vesicles, which do not refract light by PCM. We found that the cells were expressing a heterogeneous mixture of vesicles, and with a much greater proportion of smaller bicone vesicles than in wild type cells. Additionally, in large numbers of cells we observed many vesicles, but almost all were small bicones and very few were larger, mature, cylindrical structures (Figure 5b). The results suggested that the cells lacking gas vesicles (as scored by PCM) still produced copious small bicone vesicles and very few mature vesicles.

Overexpression of GvpG arrests cell growth and diminishes viable count

When examining overexpression of individual gas vesicle-associated proteins on plates, we noted that after only one day there was less growth when GvpG production was induced. Furthermore, after two days, the patches on plates appeared punctate (Figure 2). To examine how cell growth was affected and to quantify the phenotype, we grew cells carrying *gvpG* on a plasmid or an empty vector control (pQE80-*oriT*) under non-inducing conditions. After four hours of growth, we split the cultures and induced expression in half of each culture by addition of 1mM IPTG. In cultures carrying the empty vector control (pQE80-*oriT*), irrespective of induction, the optical density (OD₆₀₀) of the culture continued to increase until reaching stationary phase (OD₆₀₀ ~ 2.5). However, in strains carrying *gvpG*

on a plasmid, when induced, the OD₆₀₀ of the cultures levelled off and did not increase any further. In contrast, uninduced cultures carrying the pQE80-*gvpG* plasmid reached the same density as the empty vector control (Figure 6a).

OD₆₀₀ can be a misleading measurement as it records culture turbidity and not cell viability within a culture, therefore, at each time point we also recorded viable colony forming units. In the cultures with the empty vector control, no changes were observed and the viable cell count reached $\sim 4 \times 10^9$ cells per ml, irrespective of induction. In contrast, cultures where GvpG was induced only attained less than 1×10^7 cells per ml four hours after induction compared with 1.7×10^9 cells per ml in the uninduced culture (Figure 6b).

To assess any impacts on growth and viability due to GvpG overexpression in an alternative host, we examined the effect of excess GvpG in *Escherichia coli* strain W3110. We found no significant difference in either optical density after induction or in viable counts, suggesting that the decrease in cell growth and viable colony counts upon GvpG induction was S39006-specific (data not shown).

Because there was no discernible effect on growth in *E. coli*, we postulated that the decreased growth observed upon induction of GvpG may have been dependent on gas vesicle production. A strain of S39006 deleted for the full 16.6 kb gas vesicle genetic cluster had previously been created (Ramsay et al. 2011). The same experiment described above for S39006 was then performed using the same plasmids. As in wild type S39006, we found that overexpression of GvpG severely limited cell growth, resulting in a decrease in OD₆₀₀ and in viable colony counts. No change in either OD₆₀₀ or viable colony counts was observed for the empty vector control cultures (with or without induction) or the uninduced culture

carrying pQE80-*gvpG* (Figure 6c and 6d). Thus, the change in growth in the presence of excessive GvpG was independent of gas vesicle production.

GvpG contains a single conserved domain and an acidic tail in S39006

Given the effect of GvpG overexpression on cell growth, we examined the structure of this protein further. GvpG is a small 138 amino acid protein and contains a single conserved domain (from amino acids 1-74 in GvpG_{S39006}) which has been described in other GvpG-like proteins involved in gas vesicle biosynthesis, but for which no other function has been found (pfam15020). Using PSI-BLAST, we examined NCBI for other proteins similar to GvpG. The most similar proteins were almost all within bacteria, though there is a GvpG protein in the GV gene cluster from the archaeon *H. salinarum* PHH1 which is more distantly related (See Supplemental Figure 1 from Tashiro et al (2016) for an earlier phylogenetic tree). We aligned the most closely related amino acid sequences of GvpG-like proteins from different bacterial species (Supplemental Figure 3). The alignment, revealed that the conserved domain was similar in all GvpG orthologues, though the C-terminal end of the protein varied significantly between different species. For example, GvpG_{S39006} contains a C-terminal tail of 64 amino acids comprised largely of acidic amino acids such as aspartic acid (D) and glutamic acid (E). Of the 64 amino acids in the tail of GvpG in S39006, 18 were glutamic acid and 35 were aspartic acid, together comprising 82.8% of the amino acids in the C-terminal tail of GvpG. A pronounced enrichment in acidic amino acids was also observed in the C-terminal regions of GvpG homologues in *Psychromonas ingrahamii* 37 and *Candidatus magnetoglobus multicellularis* str Araruama (Supplemental Table 1). However, not all GvpG orthologues contained a C-terminal tail enriched for acidic amino acids. For example, the tail of the *Desulfomonile tiedjei* GvpG protein contains only seven amino acids and only one is a

glutamic acid or aspartic acid residue. Furthermore, the C-terminal tail of GvpG in *Desulfobacca acetoxidans* DSM11109 is not enriched for acidic amino acids, suggesting that this tail region may not be important for the protein's function.

To explore whether the acidic tail was essential for GvpG function, we engineered a plasmid expressing a variant of GvpG without the C-terminal tail, under an inducible promoter (pQE80-*gvpG*_{domain}) and transformed S39006 with this construct. We performed the same experiments as described above, first examining gas vesicle production when the GvpG_{domain} alone was overexpressed on plates. While growth arrested when the full-length protein was expressed, no obvious difference was observed when the acidic tail was removed (Figure 7a). Neither did overexpression of the GvpG_{domain} arrest cell growth or affect viable cell counts. Therefore, this implied that the acidic tail of the protein plays an important role in the ability of GvpG to arrest cell growth in S39006 or potentially increases the protein's stability (Supplemental Figure 4).

Though the conserved domain of GvpG was unable to arrest cell growth, we also wondered if it was capable of complementing a mutation in *gvpG*. We transformed cells carrying an in-frame mutation in *gvpG* with pQE80-*gvpG*_{domain} and examined them for gas vesicle expression under different levels of induction. However, the GvpG_{domain} alone was not capable of complementing gas vesicle formation (Figure 7b). This suggested that the C-terminal portion of the protein, containing almost exclusively acidic amino acids may be important for both gas vesicle formation and also for maintaining normal cell growth, or is essential for maintaining protein stability.

Discussion

378 Gas vesicle production in S39006 is a regulated process that facilitates flotation in this
379 pathogen, though the precise physiological role of gas vesicles in the natural environment,
380 or as part of any virulence pathway, is still unclear (Ramsay et al. 2011). From our earlier
381 work, we determined that 11 open reading frames were essential for gas vesicle formation
382 in S39006 (*gvpA1*, *-A2*, *-A3*, *-F1*, *-F2*, *-F3*, *-G*, *-K*, *gvrA*, *-B* and *-C*). Mutants defective in *gvpC*
383 produced gas vesicles that exhibited increased pressure sensitivity than those of wild type
384 and mutants defective in either *gvpN* or *gvpV* produced small diamond shaped vesicles that
385 failed to develop into the larger, mature bicone structures seen in wild type cells. As we
386 were able to complement each of these mutations by expressing the corresponding protein
387 on a plasmid (Tashiro et al. 2016), we knew that the plasmid-based copies of individual
388 proteins were functional. Additionally, because we suspected that stoichiometry of the
389 individual proteins would be important for gas vesicle assembly in S39006, we were able to
390 exploit the existing genetic tools available from our earlier study to probe this question
391 further (Tashiro et al. 2016).

392 Proteomic studies of gas vesicles in other systems have demonstrated that the relative
393 amounts of individual proteins are important and that protein-protein interactions play a
394 key role in development of gas vesicles from small diamonds into mature bicones (Chu et al.
395 2011). Furthermore, a recent paper has attempted to examine the overexpression of several
396 gas vesicle proteins in *H. salinarum* to determine how perturbation of the individual
397 proteins affects gas vesicle biosynthesis (Tavlaridou et al. 2013). The latter studies involved
398 reconstitution of gas vesicle production in a non-cognate host by expressing the p-vac
399 region (the two gene cluster required for gas vesicle formation) in *Haloferax volcanii*; not a
400 natural gas vesicle producer strain (Tavlaridou et al. 2013; Englert et al. 1992). The authors

supplemented this construct with one that overexpressed individual genes in the *gvpFGHIJKLM* cluster. Using this system, they found that overexpression of GvpG, GvpH and GvpM from *H. salinarum* resulted in loss of gas vesicle formation. However, an excess of GvpF, GvpI, GvpJ, GvpK or GvpL had no obvious impacts. Some of these results are similar to those found in this study. For example, GvpM from *H. salinarum* is similar to both GvpA2 and GvpA3 from S39006 (see Tashiro *et al* 2016, Supplemental Figure 1 for a phylogenetic analysis). We also found a slight perturbation in gas vesicle formation when GvpA3_{S39006} was overexpressed, though not the complete lack of gas vesicle observed in *H. volcanii* (Tavlaridou *et al.* 2013). Additionally, though we found a dramatic change in cell physiology when we overexpressed GvpG, we did not observe any change in gas vesicle formation or flotation. One possible explanation for this disparity may be due to the acidic tail region of GvpG in S39006 or perhaps the protein is performing a completely different function in gas vesicle assembly. What is particularly interesting is that we were unable to complement gas vesicle formation in a *gvpG* mutant with the conserved domain alone, suggesting that the acidic tail does play a role in both gas vesicle formation and in cell growth or that the tail is essential for protein stability.

We were initially surprised to find that overexpression of GvpG caused a cell growth impact and > 100 fold, decline in viable colony counts. No previous reports have linked gas vesicle formation to cell division. Furthermore, work examining overexpression of GvpG from *H. salinarum* PHH1 in *Hfx. volcanii* transformants did not identify this phenotype (Tavlaridou *et al.* 2013). However, the change in cell growth upon overproduction of GvpG may occur only in native gas vesicle-producing organisms, as we were unable to replicate this phenotype in *E. coli*, the organism within which we have previously reconstituted S39006 gas vesicles

(Ramsay et al. 2011). Thus, it would be interesting to assess the impacts of overexpression of GvpG in the native gas vesicle producing strain *H. salinarum* PHH1.

It is important to note that overexpression of GvpG was not linked exclusively to gas vesicle formation. Even in an S39006 mutant where the gas vesicle cluster had been completely removed, overexpression of GvpG caused a significant growth defect. This suggests that an S39006-specific system, not present in *E. coli*, links gas vesicle formation to cell growth or division. The fact that GvpG is both essential for gas vesicle formation and linked with cell division is an interesting observation and requires further study. To our knowledge, this is the first report linking a single gas vesicle protein to cell division.

Some proteins within the S39006 gas vesicle genetic cluster are not found in *H. salinarum* PHH1, e.g. GvpV. As mentioned previously, strains defective in *gvpV* form small bicone vesicles that do not mature into the larger cylindrical vesicles, and this led us to predict it may be acting as a chaperone, facilitating the maturation of vesicle formation (Tashiro et al. 2016). Furthermore, Konopka and colleagues reported the isolation of an *Ancylobacter aquaticus* mutant that produced only bicone gas vesicles, and so a mutation disrupting *gvpV* or a mutation resulting in GvpV overproduction are possible explanations for this phenomenon (Konopka et al. 1975). The relative abundance of individual proteins in a chaperone complex is known to be important. For example, perturbation of GrpE levels, an interacting partner of the chaperone DnaK, impairs DnaK-mediated protein refolding and also leads to changes in cell morphology, resulting in filamentous cells in *E. coli* (Sugimoto et al. 2008). GrpE is believed to act by impairing FtsZ ring formation, an important stage in cell division (Sugimoto et al. 2008; Bi & Lutkenhaus 1991). Thus, it is possible an excess of GvpV functions similarly, accumulating within the cell then eventually impairing vesicle formation,

perhaps by altering protein turnover or perturbing the protein-protein interactions important in morphogenic assembly. Little is known about protein turnover during S39006 gas vesicle formation and nothing is known about how the entire gas vesicle nanostructure is turned over in this strain.

GvrA, GvrB and GvrC are all required for gas vesicle synthesis (Ramsay et al. 2011; Tashiro et al. 2016). Each of these regulatory proteins shows similarity to two component systems. These systems are usually comprised of a sensor kinase, often located at the cell membrane, and a cytosolic response regulator (Stock et al. 2000). Both GvrA and GvrC contain response regulator receiver domains, though only GvrA contains a putative HTH DNA-binding domain. Additionally, phylogeny analysis showed that GvrA and GvrC are similar to each other (29% identical; 50% similar), but this is almost exclusively within the receiver domains. The dimerization interface within the receiver domains is also perfectly conserved between the two proteins, suggesting that they may form heterodimers. Evolutionarily, duplication is thought to be one route that allowed the expansion of two component systems within bacteria (Capra & Laub 2012). However, this would lead to heterodimerization, potentially a serious problem as the two proteins diverged evolutionarily. Recently, several groups have demonstrated that heterodimerization plays an important role in integrating environmental cues. In *Streptomyces venezuelae*, the response regulators BldM and WhiI form functional heterodimers capable of integrating cues from their respective developmental pathways (Al-Bassam et al. 2014). In *E. coli*, two response regulators RcsB and RcsA can form functional heterodimers which upregulate exopolysaccharide capsule production. RcsB is also capable of forming heterodimers with the acid stress response regulator GadE (Castanié-Cornet et al. 2010), and with BglJ, leading to repression of the aryl- β ,D-glucoside (*bgl*) operon (Venkatesh

et al. 2010). Thus, through heterodimer formation, GvrC may titrate away GvrA, leading to repression of gas vesicle formation.

GvrB contains a Per-ARNT-Sim (or PAS) motif and a histidine kinase domain and a histidine kinase-like ATPase. PAS domains can be associated with signal transduction in a variety of eukaryotic and prokaryotic organisms (Henry & Crosson 2011). They have been associated with cellular response to oxygen, light, small ligands or redox potential. Some PAS-domain-containing proteins also bind haem during oxygen sensing. For example, the oxygen-sensing kinase FixL from rhizobia and the direct oxygen sensing Dos protein from *E. coli* both contain PAS domains with haem binding pockets (Gilles-Gonzalez & Gonzalez 2004; Monson et al. 1995; Delgado-Nixon et al. 2000). The PAS domain in GvrB also contains six residues characteristic of a haem pocket. Furthermore, gas vesicle production is stimulated under reduced oxygen conditions suggesting an important regulatory mechanism for sensing and responding to oxygen concentration in the local environment. The precise biochemical link between dissolved gas in the medium and gas vesicle development is unclear, but it is possible that the heme-binding pocket within GvrB is responsible for sensing and responding to intracellular dissolved O₂ levels.

There is interest in developing gas vesicles for biotechnological translation (DasSarma et al. 2014; Childs & Webley 2012; Altschul et al. 2011), and so increasing knowledge of basic gas vesicle development will assist future exploitation of these nanostructures in synthetic biology and industrial biotechnology areas. S39006 Gas vesicle production has been engineered into *E. coli* (Ramsay et al. 2011; Tashiro et al. 2016; Li & Cannon 1998). However, functional reconstitution of vesicle assembly with concomitant flotation appears to be strain-dependent in that heterologous host (data not presented). We are currently

investigating why reconstitution works in some strains and not in others. Nevertheless, this study has provided the first evidence, to our knowledge, that the relative stoichiometry of individual proteins required for gas vesicle development in S39006 is critical for full and functional morphogenesis. Furthermore, even mild perturbation of the three Gvr regulatory proteins studied here caused almost complete repression of gas vesicle formation. Finally, we have, for the first time, established a link between bacterial growth and binary fission to gas vesicle formation operating through the GvpG protein. This is an interesting observation that warrants further investigation because, conceivably, it could be pertinent to the strain variability seen in functional reconstitution in non-cognate hosts.

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511 **Figure Legends**

512 **Figure 1. Schematic representation of the gas vesicle genetic cluster in S39006.** The 16.6 kb
513 genetic cluster of S39006 is shown, each of the 19 open reading frames contained within the
514 cluster is annotated as an arrow. The genetic cluster is made up of two distinct operons, the
515 first beginning with *gvpA1* and the second with *gvrA*. Grey arrows indicate open reading
516 frames essential for wild type gas vesicle formation and black arrows indicate open reading
517 frames which are not required.

518 **Figure 2. Overexpression of individual open reading frames within the gas vesicle genetic**
519 **cluster from a plasmid in wild type S39006.** Wild type S39006 cultures, carrying pQE80-*oriT*
520 (empty vector control) or the same plasmid engineered to express the open reading frame
521 listed across the top of the panel were spotted onto LBA plates carrying the concentration
522 of inducer IPTG (indicated on the left) and incubated at 30°C. The patch morphology is
523 shown after one day (top image) or two days of growth (middle image). PCM images were
524 taken of cells from a two-day-old colony and are shown at the bottom. Scale bars on the
525 PCM images indicate 1µm.

526 **Figure 3. Flotation assays of wild type S39006 overexpressing individual open reading**
527 **frames within the gas vesicle genetic cluster.** Wild type gas vesicles carrying pQE80-*oriT*
528 with the indicated open reading frame (top) were grown in LB with varying concentrations
529 of IPTG (left) in sealed universals at 30°C for one day. Cultures were then left to settle on a
530 stable surface for 24 hours and flotation assessed. From each flotation assay cells were
531 imaged by PCM (below). Scale bars on the PCM images indicate 1µm.

Figure 4. Overexpression analysis of the three regulatory proteins in the gas vesicle

genetic cluster. (a) Schematic representation of GvrA, GvrB and GvrC. Conserved domains

were determined by analysis for Pfam matches. Receiver domains are indicated by

rectangles, the AAA+ ATPase domain is indicated as a black rounded rectangle, the HTH

domain is indicated as an oval; the PAS domain as a star; the histidine kinase domain in grey

and the histidine kinase-like ATPase in GvrB is found in black. Above each domain, the start

and ending amino acid numbers are indicated. **(b-c)** Expression of a *gvpA1::uidA* reporter

fusion under aerobic **(b)** or microaerophilic **(c)** conditions after 16 hours of growth. Wild

type S39006 carrying the pQE80-*oriT*, pQE80-*gvrA*, pQE80-*gvrB* or pQE80-*gvrC* with the

indicated concentration of IPTG. Values represent the average of three biological replicates

and the error bars indicate +/- SD. **(d)** Prodigiosin production of wild type S39006 carrying

the plasmid pQE80-*oriT*, pQE80-*gvrA*, pQE80-*gvrB* or pQE80-*gvrC*. Values represent average

prodigiosin production after 16 hours of growth in LB for three biological replicates +/-

standard deviation.

Figure 5. Physiology of gas vesicles in strains overexpressing GvpC or GvpV. (a) Wild type

S39006 carrying either pQE80-*oriT* or pQE80-*gvpC* were grown in LB under inducing (1mM

IPTG) on non-inducing (no IPTG) conditions for 24 hours in sealed universals. Cultures were

then left to settle for 24 hours on the bench and the proportion of gas vesicle present was

ascertained over increasing incremental pressure. Data shown are the mean values +/-

standard deviation for three biological replicates. **(b)** Representative TEM images of S39006

cells overexpressing GvpV. The scale bars indicate 500 nm.

Figure 6. Overexpression of GvpG during a growth curve in wild type and GV⁻ S39006. (a)

OD₆₀₀ over 11 hours of growth of wild type S39006 carrying either pQE80-*oriT* or pQE80-

gvpG. After 4 hours, the cultures were split and IPTG (final concentration 1mM) was added to half. **(b)** At each point after IPTG induction, cells were diluted and colony counts were assessed. The same experiment was also conducted in GV- S39006 carrying the same plasmids. OD₆₀₀ was assessed over 14 hours **(c)** and viable colony counts **(d)** were assessed throughout the growth curve. Values indicate the mean +/- standard deviation for three biological replicates (error bars are on top of the marker for some time points).

Figure 7. Examination of the GvpG conserved domain. (a) Growth and gas vesicle formation of wild type S39006 carrying pQE80-*oriT*, pQE80-*gvpG* or pQE80-*gvpG*_{domain} was assessed on LBA with the indicated concentration of IPTG (left). Cells were examined after one day of growth (top), two days of growth (middle) and PCM images were taken of cells from the plate (bottom). **(b)** Gas vesicle complementation of a *gvpG* mutant on LBA with the indicated concentration of IPTG. Patch morphology (top image) of a normalized amount of culture from an in-frame *gvpG* mutant carrying either pQE80-*oriT*, pQE80-*gvpG* or pQE80-*gvpG*_{domain} after two days of incubation and representative PCM image (bottom image) of cells from the plate. For all PCM images, the scale bar indicates 1 μm.

Supplemental Figure Legends

Supplemental Figure 1. Overexpression of individual open reading frames within the gas vesicle genetic cluster from a plasmid in wild type S39006. Wild type S39006 cultures, carrying pQE80-*oriT* (empty vector control) or the same plasmid engineered to express the open reading frame listed across the top of the panel were spotted onto LBA plates carrying the concentration of inducer IPTG (indicated on the left) and incubated at 30°C. The patch morphology is shown after five days of growth (top image). PCM images were taken of cells

from the same patch and are shown at the bottom. Scale bars on the PCM images indicate 1µm.

Supplemental Figure 2. Comparative analysis of GvrA, GvrB and GvrC. (a) Clustal Omega alignment of the amino acid sequences of GvrA and GvrC. The conserved dimerization interface is indicated with a red box. A * underneath indicates a perfect match, a : indicates a conservation between highly similar groups and . indicates conservation between weakly similar groups. (b-d) Phylogenetic analysis of proteins closely related to GvrA (b), GvrB(c) and GvrC (d) from the indicated organisms, trees were generated using NJPlot and relative scales of distances are show underneath the protein name.

Supplemental Figure 3. ClustalOmega alignment of putative GvpG homologues from a range of organisms. The conserved GvpG domain (pfam05120) begins at the first amino acid and continues up to the arrow indicated above. The red colour indicates highly conserved residues and the blue indicates less conserved residues and the arrow indicates the end of the conserved domain region.

Supplemental Figure 4. Analysis of GvpG_{domain} induction throughout growth in S39006. OD₆₀₀ (a) and viable colony counts (b) over 24 hours of growth of wild type S39006 carrying either pQE80-*oriT* or pQE80-*gvpG_{domain}*. After 4 hours, the cultures were split and IPTG (final concentration 1mM) was added to half. Average values are indicated +/- standard deviation for three biological replicates (error bars are on top of the marker for some time points).

597 References

- 598 1. Pfeifer, F. Distribution, formation and regulation of gas vesicles. *Nat Rev Microbiol* **10**,
599 705–715 (2012).
- 600 2. Walsby, A. E. Gas vesicles. *Microbiol Rev* **58**, 94–144 (1994).
- 601 3. Hechler, T. & Pfeifer, F. Anaerobiosis inhibits gas vesicle formation in halophilic
602 Archaea. *Mol Microbiol* **71**, 132–145 (2009).
- 603 4. Pfeifer, F., Gregor, D., Hofacker, A., Plosser, P. & Zimmermann, P. Regulation of gas
604 vesicle formation in halophilic archaea. *J Mol Microbiol Biotechnol* **4**, 175–181 (2002).
- 605 5. Tashiro, Y., Monson, R. E., Ramsay, J. P. & Salmond, G. P. C. Molecular genetic and
606 physical analysis of gas vesicles in buoyant enterobacteria. *Environ. Microbiol.* (2016).
607 doi:10.1111/1462-2920.13203
- 608 6. Ramsay, J. P., Williamson, N. R., Spring, D. R. & Salmond, G. P. A quorum-sensing
609 molecule acts as a morphogen controlling gas vesicle organelle biogenesis and
610 adaptive flotation in an enterobacterium. *Proc Natl Acad Sci U S A* **108**, 14932–14937
611 (2011).
- 612 7. Bright, D. I. & Walsby, A. E. The relationship between critical pressure and width of
613 gas vesicles in isolates of *Planktothrix rubescens* from Lake Zürich. *Microbiology* **145**,
614 2769–2775 (1999).
- 615 8. Dunton, P. G. & Walsby, A. E. The diameter and critical collapse pressure of gas
616 vesicles in *Microcystis* are correlated with GvpCs of different length. *FEMS Microbiol.*
617 *Lett.* **247**, 37–43 (2005).

- 618 9. Arnoldi, M. *et al.* Bacterial turgor pressure can be measured by atomic force
619 microscopy. *Phys Rev E Stat Phys Plasmas Fluids Relat Interdiscip Top.* **62**, 1034–1044
620 (2000).
- 621 10. Beard, S. J., Hayes, P. K., Pfeifer, F. & Walsby, A. E. The sequence of the major gas
622 vesicle protein, GvpA, influences the width and strength of halobacterial gas vesicles.
623 *FEMS Microbiol Lett* **213**, 149–157 (2002).
- 624 11. Strunk, T. *et al.* Structural model of the gas vesicle protein GvpA and analysis of GvpA
625 mutants in vivo. *Mol Microbiol* **81**, 56–68 (2011).
- 626 12. Sivertsen, A. C., Bayro, M. J., Belenky, M., Griffin, R. G. & Herzfeld, J. Solid-State NMR
627 Evidence for Inequivalent GvpA Subunits in Gas Vesicles. *J. Mol. Biol.* **387**, 1032–1039
628 (2009).
- 629 13. Ezzeldin, H. M., Klauda, J. B. & Solares, S. D. Modeling of the major gas vesicle
630 protein, GvpA: from protein sequence to vesicle wall structure. *J Struct Biol* **179**, 18–
631 28 (2012).
- 632 14. Dunton, P. G., Mawby, W. J., Shaw, V. A. & Walsby, A. E. Analysis of tryptic digests
633 indicates regions of GvpC that bind to gas vesicles of *Anabaena flos-aquae*.
634 *Microbiology* (2006). doi:10.1099/mic.0.28755-0
- 635 15. Buchholz, B. E., Hayes, P. K. & Walsby, A. E. The distribution of the outer gas vesicle
636 protein, GvpC, on the *Anabaena* gas vesicle, and its ratio to GvpA. *J. Gen. Microbiol.*
637 **139**, 2353–2363 (1993).
- 638 16. Offner, S., Wanner, G. & Pfeifer, F. Functional studies of the *gvpACNO* operon of

- 639 *Halobacterium salinarium* reveal that the GvpC protein shapes gas vesicles. *J.*
640 *Bacteriol.* **178**, 2071–2078 (1996).
- 641 17. Holland, D. P. & Walsby, A. E. Digital recordings of gas-vesicle collapse used to
642 measure turgor pressure and cell-water relations of cyanobacterial cells. *J. Microbiol.*
643 *Methods* **77**, 214–224 (2009).
- 644 18. Williamson, N. R., Fineran, P. C., Leeper, F. J. & Salmond, G. P. C. The biosynthesis and
645 regulation of bacterial prodiginines. *Nat. Rev. Microbiol.* **4**, 887–99 (2006).
- 646 19. Coulthurst, S. J., Barnard, A. M. L. & Salmond, G. P. C. Regulation and biosynthesis of
647 carbapenem antibiotics in bacteria. *Nat Rev Micro* **3**, 295–306 (2005).
- 648 20. Hsieh, H.-Y. *et al.* Prodigiosin down-regulates SKP2 to induce p27(KIP1) stabilization
649 and antiproliferation in human lung adenocarcinoma cells. *Br. J. Pharmacol.* **166**,
650 2095–108 (2012).
- 651 21. Papireddy, K. *et al.* Antimalarial activity of natural and synthetic prodiginines. *J. Med.*
652 *Chem.* **54**, 5296–306 (2011).
- 653 22. Marchal, E. *et al.* Synthesis and antimalarial activity of prodigiosenes. *Org. Biomol.*
654 *Chem.* **12**, 4132–42 (2014).
- 655 23. Espona-Fiedler, M. *et al.* Identification of dual mTORC1 and mTORC2 inhibitors in
656 melanoma cells: prodigiosin vs. obatoclax. *Biochem. Pharmacol.* **83**, 489–96 (2012).
- 657 24. Lu, C.-H. *et al.* Prodigiosin-induced cytotoxicity involves RAD51 down-regulation
658 through the JNK and p38 MAPK pathways in human breast carcinoma cell lines.
659 *Toxicol. Lett.* **212**, 83–9 (2012).

- 660 25. Liu, P. *et al.* Undecylprodigiosin induced apoptosis in P388 cancer cells is associated
661 with its binding to ribosome. *PLoS One* **8**, e65381 (2013).
- 662 26. Fineran, P. C., Slater, H., Everson, L., Hughes, K. & Salmond, G. P. Biosynthesis of
663 tripyrrole and beta-lactam secondary metabolites in *Serratia*: integration of quorum
664 sensing with multiple new regulatory components in the control of prodigiosin and
665 carbapenem antibiotic production. *Mol Microbiol* **56**, 1495–1517 (2005).
- 666 27. Fineran, P. C., Williamson, N. R., Lilley, K. S. & Salmond, G. P. C. Virulence and
667 Prodigiosin Antibiotic Biosynthesis in *Serratia* Are Regulated Pleiotropically by the
668 GGDEF/EAL Domain Protein, PigX. *J. Bacteriol.* **189**, 7653–7662 (2007).
- 669 28. Coulthurst, S. J., Kurz, C. L. & Salmond, G. P. C. *luxS* mutants of *Serratia* defective in
670 autoinducer-2-dependent ‘quorum sensing’ show strain-dependent impacts on
671 virulence and production of carbapenem and prodigiosin. *Microbiology* **150**, 1901–10
672 (2004).
- 673 29. Wilf, N. M. *et al.* The RNA chaperone, Hfq, controls two *luxR*-type regulators and
674 plays a key role in pathogenesis and production of antibiotics in *Serratia* sp. ATCC
675 39006. *Environ. Microbiol.* **13**, 2649–2666 (2011).
- 676 30. Williamson, N. R., Fineran, P. C., Ogawa, W., Woodley, L. R. & Salmond, G. P. C.
677 Integrated regulation involving quorum sensing, a two-component system, a
678 GGDEF/EAL domain protein and a post-transcriptional regulator controls swarming
679 and RhIA-dependent surfactant biosynthesis in *Serratia*. *Environ. Microbiol.* **10**, 1202–
680 1217 (2008).
- 681 31. Tavlaridou, S., Faist, K., Weitzel, K. & Pfeifer, F. Effect of an overproduction of

682 accessory Gvp proteins on gas vesicle formation in *Haloferax volcanii*. *Extremophiles*
683 **17**, 277–287 (2013).

684 32. Chu, L. J. *et al.* New structural proteins of *Halobacterium salinarum* gas vesicle
685 revealed by comparative proteomics analysis. *J Proteome Res* **10**, 1170–1178 (2011).

686 33. Shukla, H. D. & DasSarma, S. Complexity of Gas Vesicle Biogenesis in *Halobacterium*
687 sp. Strain NRC-1: Identification of Five New Proteins. *J. Bacteriol.* **186**, 3182–3186
688 (2004).

689 34. Mikkelsen, H., Duck, Z., Lilley, K. S. & Welch, M. Interrelationships between colonies,
690 biofilms, and planktonic cells of *Pseudomonas aeruginosa*. *J. Bacteriol.* **189**, 2411–6
691 (2007).

692 35. Jeanson, S., Floury, J., Gagnaire, V., Lortal, S. & Thierry, A. Bacterial Colonies in Solid
693 Media and Foods: A Review on Their Growth and Interactions with the Micro-
694 Environment. *Front. Microbiol.* **6**, 1284 (2015).

695 36. Englert, C. & Pfeifer, F. Analysis of gas vesicle gene expression in *Haloferax*
696 *mediterranei* reveals that GvpA and GvpC are both gas vesicle structural proteins. *J*
697 *Biol Chem* **268**, 9329–9336 (1993).

698 37. Englert, C., Wanner, G. & Pfeifer, F. Functional analysis of the gas vesicle gene cluster
699 of the halophilic archaeon *Haloferax mediterranei* defines the vac-region boundary
700 and suggests a regulatory role for the *gvpD* gene or its product. *Mol Microbiol* **6**,
701 3543–3550 (1992).

702 38. Horne, M., Englert, C., Wimmer, C. & Pfeifer, F. A DNA region of 9 kbp contains all

703 genes necessary for gas vesicle synthesis in halophilic archaeobacteria. *Mol Microbiol*
704 **5**, 1159–1174 (1991).

705 39. Sugimoto, S., Saruwatari, K., Higashi, C. & Sonomoto, K. The proper ratio of GrpE to
706 DnaK is important for protein quality control by the DnaK-DnaJ-GrpE chaperone
707 system and for cell division. *Microbiology* **154**, 1876–85 (2008).

708 40. Bi, E. F. & Lutkenhaus, J. FtsZ ring structure associated with division in *Escherichia coli*.
709 *Nature* **354**, 161–4 (1991).

710 41. Chow, K. C. & Tung, W. L. Overexpression of *dnaK/dnaJ* and *groEL* confers freeze
711 tolerance to *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **253**, 502–5 (1998).

712 42. Stock, A. M., Robinson, V. L. & Goudreau, P. N. Two-component signal transduction.
713 *Annu. Rev. Biochem.* **69**, 183–215 (2000).

714 43. Capra, E. J. & Laub, M. T. Evolution of two-component signal transduction systems.
715 *Annu. Rev. Microbiol.* **66**, 325–47 (2012).

716 44. Al-Bassam, M. M., Bibb, M. J., Bush, M. J., Chandra, G. & Buttner, M. J. Response
717 regulator heterodimer formation controls a key stage in *Streptomyces* development.
718 *PLoS Genet.* **10**, e1004554 (2014).

719 45. Castanié-Cornet, M.-P. *et al.* Acid stress response in *Escherichia coli*: mechanism of
720 regulation of *gadA* transcription by RcsB and GadE. *Nucleic Acids Res.* **38**, 3546–54
721 (2010).

722 46. Venkatesh, G. R. *et al.* BglJ-RcsB heterodimers relieve repression of the *Escherichia*
723 *coli bgl* operon by H-NS. *J. Bacteriol.* **192**, 6456–64 (2010).

- 724 47. Gilles-Gonzalez, M.-A. & Gonzalez, G. Signal transduction by heme-containing PAS-
725 domain proteins. *J. Appl. Physiol.* **96**, 774–83 (2004).
- 726 48. Monson, E. K., Ditta, G. S. & Helinski, D. R. The Oxygen Sensor Protein, FixL, of
727 *Rhizobium meliloti*: role of histidine residues in heme binding, phosphorylation, and
728 signal transduction. *J. Biol. Chem.* **270**, 5243–5250 (1995).
- 729 49. Delgado-Nixon, V. M., Gonzalez, G. & Gilles-Gonzalez, M. A. Dos, a heme-binding PAS
730 protein from *Escherichia coli*, is a direct oxygen sensor. *Biochemistry* **39**, 2685–91
731 (2000).
- 732 50. DasSarma, P. *et al.* Haloarchaeal gas vesicle nanoparticles displaying *Salmonella* SopB
733 antigen reduce bacterial burden when administered with live attenuated bacteria.
734 *Vaccine* **32**, 4543–4549 (2014).
- 735 51. Childs, T. S. & Webley, W. C. In vitro assessment of halobacterial gas vesicles as a
736 *Chlamydia* vaccine display and delivery system. *Vaccine* **30**, 5942–5948 (2012).
- 737 52. Altschul, S. F. *et al.* Recombinant gas vesicles from *Halobacterium* sp. displaying SIV
738 peptides demonstrate biotechnology potential as a pathogen peptide delivery
739 vehicle. *J. Bacteriol.* **10**, 112 (2011).
- 740 53. Evans, T. J. *et al.* Characterization of a broad-host-range flagellum-dependent phage
741 that mediates high-efficiency generalized transduction in, and between, *Serratia* and
742 *Pantoea*. *Microbiology* **156**, 240–247 (2010).
- 743 54. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of
744 image analysis. *Nat Methods* **9**, 671–675 (2012).

- 745 55. Williamson, N. R. *et al.* Biosynthesis of the red antibiotic, prodigiosin, in *Serratia*:
746 identification of a novel 2-methyl-3-n-amyI-pyrrole (MAP) assembly pathway,
747 definition of the terminal condensing enzyme, and implications for
748 undecylprodigiosin biosynthesis in *Streptomyces*. *Mol. Microbiol.* **56**, 971–89 (2005).
- 749 56. Finn, R. D. *et al.* The Pfam protein families database: towards a more sustainable
750 future. *Nucleic Acids Res.* **44**, D279–D285 (2015).
- 751 57. Sievers, F. *et al.* Fast, scalable generation of high-quality protein multiple sequence
752 alignments using Clustal Omega. *Mol. Syst. Biol.* **7**, 539 (2011).
- 753 58. Perrière, G. & Gouy, M. WWW-query: An on-line retrieval system for biological
754 sequence banks. *Biochimie* **78**, 364–369 (1996).

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756